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PCT/GB2005/000225

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(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

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NP10 8QQ

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23 JAN 2004

2. Patent application number *(The Patent Office will fill this part in)* — 0401525.1

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

LingVitae AS
Trimveien 6, A562
0372 Oslo
Norway

Patents ADP number *(if you know it)* — 8612582001

If the applicant is a corporate body, give the country/state of its incorporation — Norway

4. Title of the invention — Method of Analysis

5. Name of your agent *(if you have one)*

Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

Number of earlier UK application	Date of filing *(day / month / year)*

8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request?

Answer YES if:

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

Otherwise answer NO (See note d)

YES

Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	
Description	5
Claim(s)	1
Abstract	
Drawing(s)	1

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for a preliminary examination and search (Patents Form 9/77)	
Request for a substantive examination (Patents Form 10/77)	NO
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

For the applicant
Gill Jennings & Every

Signature John Jappy Date 23/01/04

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

JAPPY, John William Graham
020 7377 1377

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Method of Analysis

Field of Invention

The present invention relates to methods for improving polynucleotide ligation reactions.

Background to Invention

Berg and Boyer created the first recombinant DNA molecule in 1972. This simple concept of recombination - the splicing together of two pieces of DNA and fusing them by ligation, is the basis for the entire field of molecular biology. Molecular biology has become ubiquitous to the point where it is central to the majority of all biological research. The ligation reaction is performed thousands of times a day in research and diagnostic laboratories worldwide. Given the boundless opportunity presented by genetic engineering, the ligation reaction is likely to remain a central technique for many years to come.

The ligation reaction itself is chemically simple, comprising the linking of two nucleotides by the creation of a phosodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another, by a ligase enzyme. There are two types of ligation, known as "sticky end" and "blunt end", depending on the presence or lack (respectively) of complementary single stranded regions on the two polynucleotides to be joined, in proximity to the ligation location. "Sticky-end" ligations involve the hybridisation of complementary single stranded sequences between the two polynucleotides to be joined, prior to the ligation event itself. Sequences that have similar but not 100% complementary single stranded sequences will still be ligated, known as a mismatch ligation. These result in the ligation of an incorrect sequence and decrease the efficiency and fidelity of the overall ligation reaction.

Since ligation is such an important reaction, ligases are available on the market that are improved, modified and optimised to give maximum efficiency. These enzymes are expensive and it is therefore desirable to use as a small amount as is possible without reducing the efficiency of the reaction and whilst avoiding mismatch ligation. Mismatch ligations are problematic as they are deleterious to the fidelity of the ligation process. It is therefore desirable to minimise mismatch ligations.

Current methods of increasing ligation specificity include decreasing the amount of ligase and increasing the salt in the reaction mix to slow down the reaction. Since match ligations are much faster than mismatch ligations, the increased specificity observed using this technique is a result of the slower reaction speed and whilst this increases the match: mismatch ratio, it results in a low yield and does not prevent mismatch ligations.

Summary of Invention

The present invention is based on the realisation that specificity in "sticky-end" ligations can be increased by including short adapters that reduce the occurrence of mismatch ligation.

According to a first aspect of the invention, a method for improving the binding between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, said second polynucleotide being present in a sample comprising a mixture of different polynucleotides, comprises:

contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution.

The present invention improves the yield of match ligations by reducing mismatch ligations through the use of blocking polynucleotides which hybridise incorrect single stranded overhangs.

Description of the Drawings

The present invention is illustrated by reference to the accompanying drawing, where:

Figure 1 is a graphic illustration of match:mismatch ratio as a function of time.

Detailed Description of the Invention

The present invention is used to increase specificity of polynucleotide ligations. The term "polynucleotide" is used herein to refer to biological molecules made up of a plurality of nucleotides. Preferred polynucleotides include DNA, RNA and synthetic analogues thereof, including PNA.

The term "hybridising conditions" is used herein to refer to conditions that allow complementary base pairing to occur between two polynucleotides, such that two complementary single stranded polynucleotides will hybridise to form a duplex. Such conditions are well known in the art. An Example of such conditions is incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

In any ligation reaction, two polynucleotide molecules are joined. The term "first polynucleotide" is used herein to refer to one of the two intended targets of ligation. The term "second polynucleotide" is used herein to refer to the other of the two intended targets of ligation.

A non-limiting example of the terms "first polynucleotide" and "second polynucleotide" comprises the "first polynucleotide" being a DNA vector into which an insert, the "second polynucleotide", is to be ligated to form a recombinant construct.

In any ligation reaction, there may be polynucleotides present which are neither "first polynucleotides" or "second polynucleotides". These polynucleotides interfere with the ligation between the first and second polynucleotides, which results in mismatch ligations. As used herein, the term "third polynucleotide" is used to describe polynucleotides which are added to the ligation reaction mixture to hybridise to any polynucleotide which is not a first or second polynucleotide, preventing the unwanted polynucleotides from reacting with the other components of the reaction mix. The third polynucleotides are not totally complementary to the first or second polynucleotides.

The method increases specificity in polynucleotide ligations through the addition of one or more third polynucleotide(s) into a reaction mix. This reaction mix comprises a first polynucleotide and a second polynucleotide, which contain complementary single stranded portions. The second polynucleotide is present in a sample comprising a mixture of different polynucleotides, of which only one ("the second polynucleotide") is the correct target for binding to the first

polynucleotide. The third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base. The number of differences between the first and third polynucleotides may depend on the size of the single stranded portions involved. For example, if the single stranded portion is only 3 bases in length, a single difference may be suitable, but if the single stranded portion is 6 bases in length, multiple differences may be preferred. The differences may be substitution(s), deletion(s) or addition(s).

The third polynucleotide may be added to the sample containing the second polynucleotide simultaneously with or sequentially before or after the first polynucleotide. The third polynucleotide is preferably added to the sample containing the second polynucleotide, along with the first polynucleotide.

Preferably, the third polynucleotide is present in excess with respect to the first and second polynucleotides, to ensure that all other polynucleotides in the sample are hybridised by the third polynucleotide.

It is intended that the first and second polynucleotides hybridise and are ligated together, to the exclusion of other polynucleotides in the sample. The third polynucleotide(s) hybridise to the other polynucleotides in the sample which would otherwise compete for binding to the first polynucleotides, effectively preventing them from hybridising to the first polynucleotides and increasing the number of correct binding events between the first and second polynucleotides. This increases the efficiency and yield of the overall ligation reaction.

Preferably the mixture of third polynucleotides comprises double stranded polynucleotides with a single stranded portion, such that the single stranded portion hybridises its complementary region on incorrect first and second target polymers.

Preferably, the single stranded portion of each of the first, second and third polynucleotides is from 3 to 6 bases in length. Most preferably, the single stranded portion is 4 bases in length.

Figure 1 is a graphical representation of the match:mismatch ratio as a function of time. This ratio becomes lower as the reaction progresses, since the match reaction rapidly reaches plateau and is caught up by the slower mismatch

reaction. Traditional methods of increasing specificity merely slow the reaction (using less ligase or increasing salt concentration) and shift the reaction to the left of the graph, where the match:mismatch ratio is favourable but yield is decreased. The present invention ensures that the mismatch ligations do not increase. The match reaction can proceed to full term without the mismatch reaction ever catching up. This gives improved yield and optimised match:mismatch ratio.

CLAIMS

1. A method for improving the binding between a first double stranded polynucleotide having a single stranded portion and a second polynucleotide having a complementary single stranded portion, said second polynucleotide being present in a sample comprising a mixture of different polynucleotides, comprising:

contacting the sample, under hybridising conditions, with the first polynucleotide and one or more third polynucleotide(s), wherein the third polynucleotide(s) comprises a single stranded portion that differs from the single stranded portion of the first polynucleotide by at least one base substitution.

2. A method according to claim 1, wherein the third polynucleotide is a double stranded polynucleotide having said single stranded portion.

3. A method according to claim 1 or claim 2, wherein the single stranded portion on each of the first, second and third polynucleotides is from 3 to 6 bases in length.

4. A method according to claim 3, wherein the single stranded portion is 4 bases in length.

5. A method according to any previous claim, wherein the single stranded portion of the third polynucleotide differs from the single stranded portion of the first polynucleotide by one base.

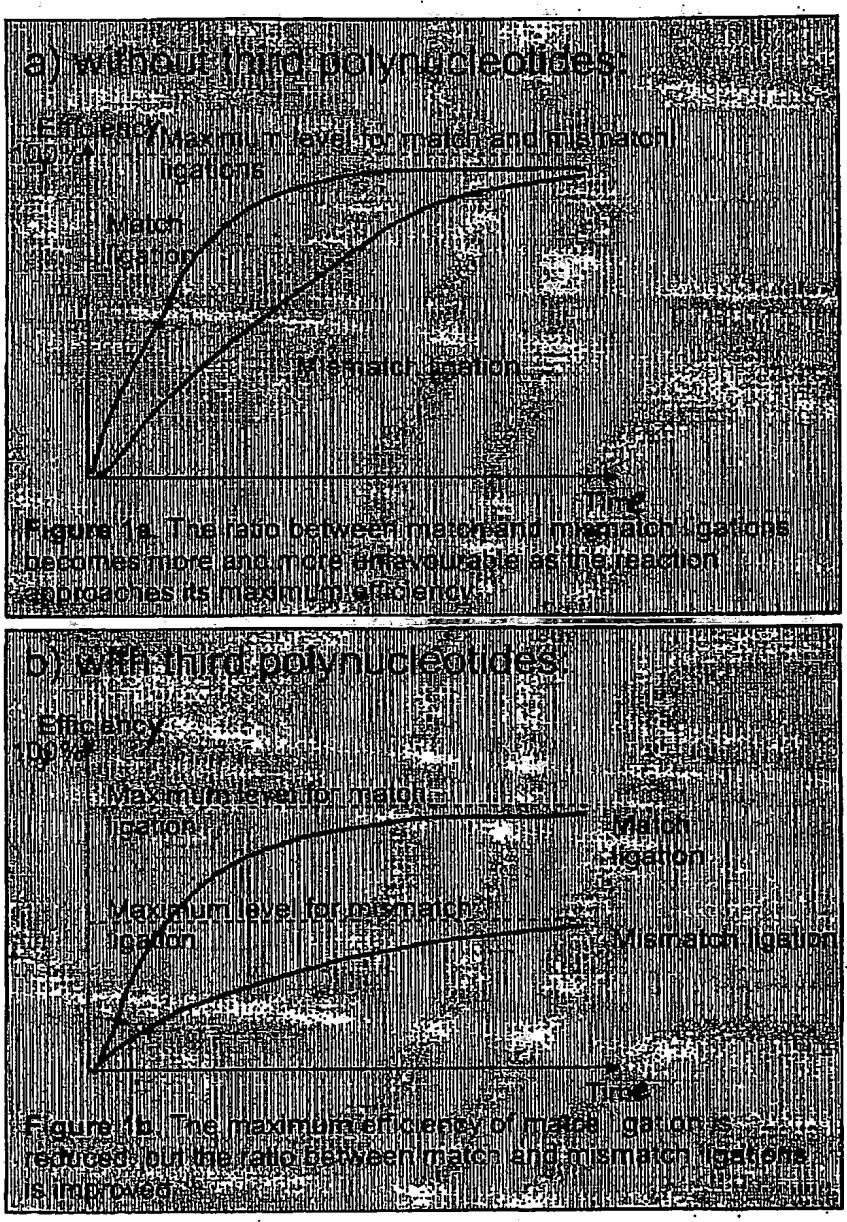
a) without third polynucleotides:
Efficiency
100%
Maximum level for match and mismatch ligations
Match ligation
Mismatch ligation
Time
Figure 1a. The ratio between match and mismatch ligations becomes more and more unfavourable as the reaction approaches its maximum efficiency.
b) with third polynucleotides:
Efficiency
100%
Maximum level for match ligation
Maximum level for mismatch ligation
Match ligation
Mismatch ligation
Time
Figure 1b. The maximum efficiency of match ligation is reduced, but the ratio between match and mismatch ligations is improved.

Figure 1